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FOREWORD

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A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY would require a further increase in the subscription price or, alternatively, would reduce the number of pages which could be assigned to other authors who desire to publish articles relating to the many fields of physiology encompassed by this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

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DETLEV W. BRONK

STUDIES ON THE PHYSIOLOGY OF FROZEN PLANTS AND ANIMALS IN THE ARCTIC

BY

P. F. SCHOLANDER, WALTER FLAGG,
R. J. HOCK, AND LAURENCE IRVING

Experimental work conducted at the
ARCTIC RESEARCH LABORATORY
POINT BARROW, ALASKA

STUDIES ON THE PHYSIOLOGY OF FROZEN PLANTS AND ANIMALS IN THE ARCTIC[1]

P. F. SCHOLANDER,[2] WALTER FLAGG,[3] R. J. HOCK[4]
AND LAURENCE IRVING[4]

TWELVE FIGURES

Few events in nature are more stirringly spectacular than the advent of spring after an arctic winter. Willows start blooming through the snow and are visited by bumblebees, while freezing temperatures are still frequent. Larvae crawl over the snow, and in a few weeks the tundra is abloom and buzzing with insects. Shallow ponds which had been frozen solid melt and soon swarm with aquatic life. Some of the coldest places on earth, in Canada and Siberia, support a lush forest and a rich fauna, which thrive in spite of winter temperatures often as low as − 50° to − 60°C. The rich alpine floras and faunas in southern latitudes offer another striking illustration of how a multitude of different forms of life have adapted to survive temperatures far below freezing.

It is a common observation that twigs and branches of bushes and trees get brittle in the cold, and the presence of crystal masses or sheets of ice in the twigs, buds, and roots can readily be ascertained by inspecting the broken surfaces. It has long been known that overwintering larvae of arctic insects freeze brittle during the winter. This is also true for insects in the cold winter of temperate climates.

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What makes this winter survival possible is a question of prime practical importance in agriculture, and the studies of cold hardiness in cultivated plants and in insects have produced a very considerable literature. There are fortunately several very excellent reviews: Chandler '13, Payne '26, Åkerman '27, Maximov '29, Cameron '30, Harvey '36, (3620 references!), Luyet and Gehenio '40, Levitt '41 and '51, and Scarth '44.

During the winter of 1948 a series of investigations was made at the Arctic Research Laboratory, Point Barrow (latitude 71°), Alaska, on the survival and physiology of frozen plants and animals. We shall deal with the following topics as bearing upon survival in the frozen state:

I. Material and microclimatic conditions.
II. Moisture content in overwintering plants and insects.
III. Ice formation in a living chironomid larva and a lichen.
IV. Respiration of frozen organisms.
V. Effects of freezing arctic plants at liquid oxygen temperature.
VI. Ice as a habitat for hibernating organisms.
    1. Estimation of solubility of air in ice.
    2. Estimation of diffusion of gases through ice.
    3. Mechanical effects of freezing in ice.
VII. Experiments on freezing the blackfish *(Dallia pectoralis)*.

We shall refer to specific literature under each of these headings.

I. MATERIAL AND MICROCLIMATIC CONDITIONS

Most of the material used in this study was obtained in the vicinity of the Point Barrow laboratory. Suitable plants were located in the late autumn before the snow had covered the ground and were marked by numbered poles. Later on these plants could be dug out from under the snow, and the overwintering basal parts and roots were chopped out with an ax in a block of frozen soil, in which they were brought to the laboratory. The chironomid larvae spent the winter frozen into the first inches of muddy bottom of a shallow pool below six inches of ice, or more. With deft hands our Eskimo helpers would chop out square blocks of ice, with two to three inches of frozen mud adhering to them. Some chironomids and crustaceans were obtained from ice blocks removed from the dense growth of aquatic grass *(Arctophila fulva)*. These blocks with the grass or mud frozen in them were sawed into two-inch thick, partly transparent ice slices and stored outside the laboratory, where the temperature was between — 10° and — 40°C.

TABLE 1

Temperature of bore holes in bare tundra near Point Barrow January, 1948. Wind estimated 40 m.p.h.

	AIR TEMP.	LOCALITY I Depth			LOCALITY II Depth	
		5 cm	12 cm	25 cm	18 cm	25 cm
	°C.					
Jan. 30	—24.0	—22.8	—22.6	—22.2	—24.6	—24.5
Jan. 31	—17.0	—19.7	—20.0	—20.5	—19.5	—19.8

TABLE 2

Temperature in snow drifts near Point Barrow, January, 1948

DEPTH IN SNOW	0 cm	1 cm	10 cm	20 cm	50 cm	100 cm
	°C.					
January 24	—28.2	—28.7	—28.6	—25.3	—20.2	—19.2
January 25 (sunny)	—7.6	—10.5	—19.6	—20.8	—20.9	—19.0

A series of temperatures on and in the ground near the Barrow camp was taken on tundra blown practically free from snow (table 1), and bearing sparse vegetation. In table 2 are given temperatures at various depths in the snow. Table 3 gives the ice and mud temperatures in the shallow pool, “Lepidurus pond,'' from which we got most of the chironomids. It will be seen that during the winter all these habitats encounter temperatures down to around — 20°C., probably for several months at a time.

TABLE 3

Temperature in bottom-frozen ''Lepidurus Pond'' ¼ mile southwest of Point Barrow camp, January 29, 1948. Depth of snow cover, 16 cm; depth of ice, 36 cm

AIR TEMP.	IN ICE				IN MUD	
	0 cm	13 cm	24 cm	36 cm	5 cm	14 cm
°C.						
— 35.4	— 24.3	— 22.5	—20.6	— 20.6	— 22.8	— 20.7

TABLE 4

Air temperature (°C.) at Point Barrow (B), Umiat (U), and Fairbanks (F), Alaska, 1950–1951 [1]

	October			November			December		
	B	U	F	B	U	F	B	U	F
Minimum	—18	—25	—24	—23	—33	—40	—31	—46	—35
Average	—6	—10	—3	—9	—12	—21	—23	—28	—21
Maximum	1	2	17	3	5	0	—9	—4	—2

	January			February			March			April		
	B	U	F	B	U	F	B	U	F	B	U	F
Minimum	—47	—52	—48	—47	—53	—42	—40	—46	—41	—27	—32	—13
Average	—33	—39	—31	—30	—34	—22	—27	—33	—18	—20	—16	1
Maximum	—14	—11	—12	—10	—3	4	—18	—22	11	2	4	16

At the subarctic base at Umiat, 100 miles south of Barrow, we obtained material from willow and alder. These bushes project above the snow and are subjected to very cold winter temperatures, often well below — 40°C. Experiments were also run on twigs from several trees at Fairbanks, which is well known for its cold winters (table 4).

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[1] We wish to express our gratitude to Robert Dale, Climatologist, U. S. Weather Bureau, Anchorage, for helping to compile these data.

II. MOISTURE CONTENT IN OVERWINTERING PLANTS AND INSECTS

There is much evidence to indicate that a certain degree of dehydration may increase the cold hardiness of plants and animals. Shutt ('03) reported that shoots of hardy apples had lower water content in the winter than had the less hardy kinds. Chandler ('13) found that peach buds, if slowly dried, increased their hardiness considerably. Tysdal ('33) observed that wilted alfalfa survived freezing much better than normally watered alfalfa. Scott and Cullinan ('46) subjected peach shoots to different degrees of dehydration and found less injury by freezing the more the dehydration.

In other cases dehydration does not seem to play an essential role in cold hardiness. Hildreth ('26) found no correlation between the hardiness of apple varieties and the moisture content of the twigs. The moisture content, however, showed a seasonal variation, with lowest values in the winter. J. H. Martin ('27) found that the hardy wheat plants contained less moisture than the non-hardy types. Rye, however, tended to have a high moisture content, although extremely hardy. In the pitch pine *(Pinus rigida)* Meyer ('28) reported that the total water content of the hardened winter leaves was not materially less than in the summer. Ullrich ('43) discussed the fact that *Stellaria media* and *Urtica urens* stand freezing brittle at temperatures of —9° to —12° and that *Helleborus* survives —17°, even though fully turgescent.

In the animal kingdom dehydration is often a factor in cold resistance. Bachmetjew (1899) found that the freezing temperatures of several insects became lower the lower the water content. Tower ('06) reported that the potato beetle in preparing for winter lost 27% of its water. Payne ('27a and b) found in two species of oak borers that dehydration promoted hardiness by lowering the freezing point and the undercooling point. She also found ('27b) that several species of insects, including the oak borers, prepared for hibernation by self-dehydration. Holmquist ('28) observed that the water content of the ant *Formica ulkei* went from an average of 76% in the summer to 61% in the winter hibernation.

At extremely low temperatures dehydration seems to be essential for survival. Becquerel ('32a) found that tuberous roots of a commercial buttercup *(Ranunculus asiaticus?)*, when air dried until the moisture content was reduced to 9.6%, would survive 18 days in liquid nitrogen (— 190°). If first moistened they were killed. Similar results were obtained with several dry seeds ('32b). In 1948[5] he reported experiments performed on microscopic algae and rotifers adhering to the lichen *Xanthoria parietina*. He kept one lot of the material normally dry, a second lot dried and partially evacuated over calcium chloride, and a third lot highly evacuated. Upon testing the material after six years, the normally dry lot had lost much of its vitality, but the others were still fully viable. He subjected the samples to two weeks' chilling in liquid air and found no impairment of survival in the artificially dried samples. In later experiments ('50, '51) he subjected tardigrades, rotifers, and the lichen *Xanthoria* to temperatures within a fraction of a degree of absolute zero, using an adiabatic demagnetization technique in high vacuum. Some of these had been under vacuum for eight years and had been for two weeks in liquid air. They survived, and similarly algae, lichens, and mosses were reported to withstand chilling to near absolute zero. Mosses with 60–80% water were chilled slowly or rapidly to liquid air temperature and kept there for seven days. He reported that they came out with "all cells" alive. As far as the survival of the animals is concerned, there can be no doubt about the conclusiveness of these experiments. As to the lichens, it seems unconvincing that microscopy can settle whether they were viable or not.

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[5] In this and an earlier thought-provoking paper ('36) Becquerel discusses the possibility of cosmic survival of such forms as have shown viability in the laboratory after complete desiccation and extreme chilling, and he mentions the possibility of finding viable forms, for instance, in or under the polar ice caps. It is interesting to note that Arrhenius ('08) devotes a chapter of his book, "Worlds in the Making," to a discussion of the physical possibility for the transport of desiccated living spores through the universe by electrostatic forces and radiation pressure. Whether the radiation would be lethal under these conditions does not seem to be known. With rocket exploration of outer space already within reach, the problem of cosmic survival becomes increasingly significant.

Locket and Luyet ('51) found that air-dried wheat seeds retained full germination (92%) after being in liquid nitrogen for two minutes, whereas they all died if first soaked to a moisture content in the embryo of 50%. The air-dried embryo contained only about 10%. Rotifers and tardigrades are well-known examples of animals that can be chilled in liquid helium without harm, if first dehydrated (cf. Luyet and Gehenio, '40).

From the examples above it seems clear that the state of hibernation is often associated with dehydration. In general, the major factor in killing by frost is not the temperature *per se*, but the ice formation, supercooling being essentially harmless. Ice formation in hardy species practically always starts in the extracellular spaces (Müller-Thurgau, 1880). As the extracellular ice masses grow the extracellular fluid concentrates and consequently more and more water is drawn from the cells by osmosis (cf. Nilsson-Leissner, '29). If this cell dehydration proceeds too far the protoplasm may suffer irreversible coagulation, and the cell dies. Intracellular ice formation is believed to be nearly always fatal, even in cells which can endure extremely low temperatures (cf. Scarth, '44).

The essence of cold hardiness seems to be the avoidance of intracellular protoplasmic ice formation. Such ice formation may be opposed by osmotic and colloidal forces, and by supercooling. Increased sugar formation and dehydration both increase the osmotic pressures, and are regarded as auxiliary to the more fundamental protection against freezing inherent in specific protoplasmal properties (Scarth, '44; Levitt, '51). Levitt and Scarth ('36) demonstrated an increase in cell permeability to water correlated with hardiness. This facilitates a rapid loss of water from the cells to the extracellular spaces, preventing intracellular ice. Kessler and Ruhland ('42) found increased water binding in the protoplasm of winter-hardened *Sempervivum* leaves and *Catalpa* bark. This would protect against cytoplasmic ice formation and would prevent a dangerous inundation of the plasma with water on thawing. Simonovitch and Briggs ('49) found that the amount of water-soluble proteins in the bark of the black locust tree *(Robinia pseudoacacia)* was closely correlated with the cold hardiness, in contrast to the amount of sugars. They suggested that the presence of these hydrophilic proteins may not only resist dehydration, but may also prevent intracellular ice formation by favoring supercooling.

The above investigations indicated that as a rule a high degree of dehydration is a prerequisite for survival at extreme temperatures, whereas, in moderate cold, it is seemingly a factor of less importance. It was of interest, therefore, to see to what extent dehydration appears as a factor in the survival of hibernating arctic forms.

Procedure and results

A series of rhizomes, with fresh roots adhering to them, were collected in the middle of August, while the plants were in full vigor. Soil, withered leaf and root sheaths, and other dead parts were removed. Most often a quick rinsing in water and immediate drying off with filter paper were necessary before the weighing. The errors introduced by the wetting were somewhat counteracted by the tendency towards water loss during the preparation. In the winter (February–March) the same parts were chopped out of the ground with an ax and shaved as free as possible from adhering material while still frozen. They were then taken into the laboratory, and given a quick thawing and rinsing off in water. They were immediately dried off with filter paper, and dry and dead parts were removed. Live weight was taken, after which the material was kept in a drying oven at 100° overnight. Thick parts were split open to promote drying.

It will be seen from table 5 that 7 out of 8 species of plants clearly showed dehydration in the winter. At what stage of the hardening process this occurred we do not know. It may well be a consequence of the freezing. Although not too striking, dehydration may be considered a contributing factor towards cold hardiness in many arctic plants.

Observations were made upon the moisture content of one species of insect, namely, a red chironomid larva, living in the mud of a small shallow pool near the laboratory. When melted out of the frozen mud by means of a gentle jet of cold tap water, the larvae were wrinkled and without turgor, and hence visibly dehydrated. They were immediately weighed, then placed in water until normally active, and reweighed. Within 6 hours most had reached full hydration, but some gained further weight in the next 18 hours. The weight increase varied between 10 and 20%. However, even completely hydrated, feeding larvae were frozen at — 20° or lower and

TABLE 5

Ratio between water and dry weight in living basal parts and roots of arctic vascular plants in summer and winter

	AUGUST	AVER.	FEBRUARY–MARCH	AVER.	WINTER WATER IN % OF SUMMER WATER
Poa arctica R. Br.	1.8,1.5,1.9, 2.3,2.3	1.96	0.69,0.89,0.70, 0.60,0.62	0.70	36
Luzula nivalis (Laest.) Beurl.	2.6,2.7,1.8, 2.6,2.8	2.53	1.5,1.4,1.8, 1.5,1.4	1.53	60
Eriophorum polystachyum Honck.	3.3,2.9,3.0, 3.9	3.25	2.0,2.1,1.8, 2.3,2.2	2.09	64
Salix rotundifolia Trautv.	1.4,2.1,1.2, 1.3	1.51	0.85,0.76,0.49, 0.68,0.78	0.71	47
Papaver radicatum Laest.	2.4,2.3,2.3, 2.2,2.6	2.33	2.6,2.5,2.3, 2.4,2.5	2.43	104
Potentilla emarginata Pursh.	1.8,1.7,1.7	1.74	1.5,1.2,1.4, 1.2,1.1	1.29	74
Pedicularis sudetica Willd.	3.2,3.6, (5.8)	3.41	2.7,2.5,2.7, 2.9,2.4	2.62	77
Petasites frigidus (L) Fries	3.2,2.9,3.8, 3.6,2.6	3.21	0.68,0.82,0.74, 1.2,0.81	0.84	26

thawed several times without harm. We do not know whether a dehydrated larva will stand lower temperatures than a fully hydrated one.

Eggs, presumably of *Cyclops,* were observed to be caved in and wrinkled, and hence visibly dehydrated, while in the ice. After thawing they swelled out round within a few hours. The alga colonies of *Nostoc* likewise came out of the ice dehydrated, and rounded off after several hours in water.

At the end of May, 1948, some tussock moth larvae, *Birdia (Laria) rossi,* were found crawling on the snow, presumably therefore normally hydrated. One of these was left at — 10° overnight and revived in half an hour the next day when thawed out. It spent the next 28 hours at — 15° and survived.

It seems that many animals that freeze in the ice or in the air are likely to become somewhat dehydrated as a result of the freezing process. Some, however, dehydrate before the freezing. In other cases survival is possible although dehydration does not precede the freezing. If dehydration is a major factor in the survival of these forms at extreme temperatures, it must be sought on a cellular level.

III. ICE FORMATION IN A LIVING CHIRONOMID LARVA AND A LICHEN

It was found that the chironomid larvae, like the overwintering plants, were usually some 10% dehydrated, which means that they still passed the winter with some 60–70% water in their tissues. The temperature where these forms overwinter stays around — 20° for several months. For many other forms which spend the winter exposed, the temperature may reach — 50° or lower.

We may wonder what happens to the water in the insects and plants at these temperatures. We have undertaken to measure quantitatively the amount of ice present at different temperatures in a chironomid larva and in a lichen.

Ice formation in the twigs and buds of temperate plants is of regular occurrence. Wiegand ('06) demonstrated this in 27 species of trees and shrubs examined around Ithaca, New York. At — 18° all but eight had ice in the buds, and at — 23.5° only four remained without ice. There was a marked correlation between the amount of ice found and the total moisture content. The bud with the lowest moisture content froze at the lowest temperature. Around Fairbanks we observed ice regularly in roots and twigs of forest trees, and likewise in shrubs and roots of plants around Point Barrow.

Many insects will also survive having their body fluids frozen. This must be of regular occurrence in many arctic species, although direct observations of the ice crystals seem to be lacking. Commander James Clark Ross, wintering at Boothia Peninsula (latitude 70°), collected many tussock caterpillars of *Birdia (Laria) rossi* in September (1831 ?). Curtis (1835) writes: "The following interesting experiments I have transcribed from Commander Ross's MSS. 'About thirty of the caterpillars were put into a box in the middle of September, and after being exposed to the severe winter temperature of the next three months, they were brought into a warm cabin, where in less than two hours, every one of them returned to life, and continued for a whole day walking about; they were again exposed to the air at a temperature of about 40° below zero, and became immediately hard frozen; in this state they remained a week, and on being brought again into the cabin, only twenty-three came to life; these were at the end of four hours put out once more into the air, and again hard frozen; after another week they were brought in, when only eleven were restored to life; a fourth time they were exposed to the winter temperature, and only two returned to life on being again brought into the cabin; these two survived the winter, and in May an imperfect *Laria* was produced from one, and six flies from the other; both of them formed cocoons, but that which produced the flies was not so perfect as the other.' " The following observation was made on another species, probably *Melitaea tarquinius:* "Commander Ross was so fortunate as to discover the caterpillar apparently of this species, from its structure resembling those larvae that are known of the

European *Melitaeae.* ' . . . one that was found under a stone in the middle of March, and of course perfectly hard frozen, showed symptoms of life in half an hour after being brought into the cabin, and in less than an hour it was walking about the table . . .' "

Deichmann (1896), while wintering in Scoresby Sound, Northeast Greenland, latitude 73½°, noticed that butterfly larvae preferentially selected high spots which were blown bare of snow for their wintering habitats. They were there frequently subjected to winds and temperatures of — 40°. The larvae *(Dasychira groenlandica, Argynnis, Colias* ?, and *Noctuidae)* were frozen stiff. Spiders *(Lycosa)* were also found in these places. Johansen ('10), wintering in Northeast Greenland, 76° 46′ north latitude, found under a tuft of *Dryas* a 3 cm *Anarta* larva frozen stiff. The plant was almost free of snow and half of the animal was completely exposed. It came to life when brought indoors, but died some ten days later.

Kapterev ('36) found that *Cyclops* and *Planorbis* would survive after being thawed out of the ice of shallow pools near the Amur River in southeastern Russia. Samples of soil taken two to four meters down in the permafrost showed growth, when thawed, of 20 genera of extant algae, and also a living moss and a crustacean, all of which were considered to have survived frozen for hundreds or perhaps thousands of years. Such an ancient origin seems unlikely in view of the fact that neither was the temperature very low (— 1.5° at 3.2 m depth) nor could the dehydration by freezing have been great enough to virtually stop the metabolic rate. Andersen ('46) chopped out a block of frozen bottom material from under 40 cm of five-month old ice in an East Greenland lake at 72° 50′ north latitude. Upon thawing there emerged nine species of live chironomids, which very likely had been frozen.

Ice formation in many temperate climate insects has been directly determined by several authors, as reviewed by Uvarov ('31) and by Luyet and Gehenio ('40). Sacharov ('30) found by dilatometric technique that the overwintering larvae of the moth *(Euproctis chrysorrhoea)* had 71.8% total water. It survived freezing at — 17.4° when 15.2% of the total water was frozen. An adult moth, *Scoliopteryx,* survived — 11.1° with 28.9% of its total water frozen. Larvae of a beetle, *Plagionotus arcuatus,* survived — 17.4° with 3.5% of the total water frozen.

Salt ('50) points out that the majority of temperate cold hardy insects will stand supercooling, but will die if frozen. Supercooling is an unstable condition, and for a given cold hardiness freezing will statistically take place sooner the colder it is. Given enough time, even a moderate temperature will lead to freezing and killing. Supercooling has ben demonstrated by the same author to be a major factor in the survival of temperate forms through the winter.

Ice formation in chironomid larvae

Chironomid larvae of different species are very common in the ponds around Point Barrow. They were found buried in the frozen black mud in shallow pools near the laboratory. The temperature taken in bore holes showed this habitat to go below — 20° (table 3). Chopped-out pieces of the frozen mud were placed outdoors near the laboratory for several months, subjected to temperatures frequently as low as — 40°. From these the larvae could be thawed out by a jet of cold tap water. The thawed-out larvae were somewhat wrinkled by dehydration, and if placed on a filter paper and put in the refrigerator at — 6° to — 10° they rapidly froze, changing from a transparent dark red to a completely opaque yellowish orange. This striking change in optical properties strongly suggested that the larvae were filled with highly refractive ice crystals. With decreasing temperature the larvae got more brittle. At — 10°, for instance, the head would easily break off when prodded. At — 32° the larvae would still be somewhat pasty, however, and even at this temperature the broken surface was observed to yield a fine drop of unfrozen dark "blood," when squeezed. When dried off on a filter paper, the larvae could be frozen and thawed repeatedly, and when put back in cold water they would in an hour or two become active and start to feed. Four larvae were frozen at — 16° and thawed in all four times. All remained alive.

As we obviously had before us an insect which, like so many plants, could stand internal ice formation, it was desirable to investigate what quantitative relation there might be between the ice formation and the temperature.

Micro flotation method for quantitative ice determination at graded temperatures

Two principles have been generally employed for the determination of the amount of ice formed in an organism upon freezing. In the calorimetric methods most commonly used, introduced by Müller-Thurgau (1886) and later developed by Thoenes ('25), Robinson ('28), Greathouse ('35), Ditman, Vogt and Smith ('42), and others, ice is determined from the heat of fusion. In the dilatometric method the expansion which takes place from the freezing of water is used as a measure of the ice formation. This was employed by Foote and Saxton ('16), Bouyoucos ('17), Moran ('35), Gortner ('37), and others.

Due to the extreme scarcity of material at Point Barrow neither of these macro methods could be used. Hence a micro flotation method was developed, taking advantage of the change in specific gravity of the larva when ice is formed and of the fact that the specific gravity of even minute objects can be determined with high accuracy by flotation. This principle has seemingly not been previously used for ice determination in biological material, but it was applied in 1862 by Dufour in the determination of the specific gravity of ice. He added chloroform to kerosene until he obtained a mixture which exactly floated a piece of clear air-free ice, whereupon he determined the specific gravity of the flotation medium.

Principle. The specimen, which must be air-free, is placed in kerosene contained in a small vial hung in a thermoregulated kerosene bath. Enough bromobenzene is added for the specimen to remain suspended in the mixture. The mixture is transferred to a pycnometer kept in the same kerosene bath. The density of the mixture — and hence of the specimen — is determined by weighing. The procedure is carried through at + 1° and at desired temperature steps below freezing. The weight percentage of ice in the specimen is calculated from the density above and below freezing.

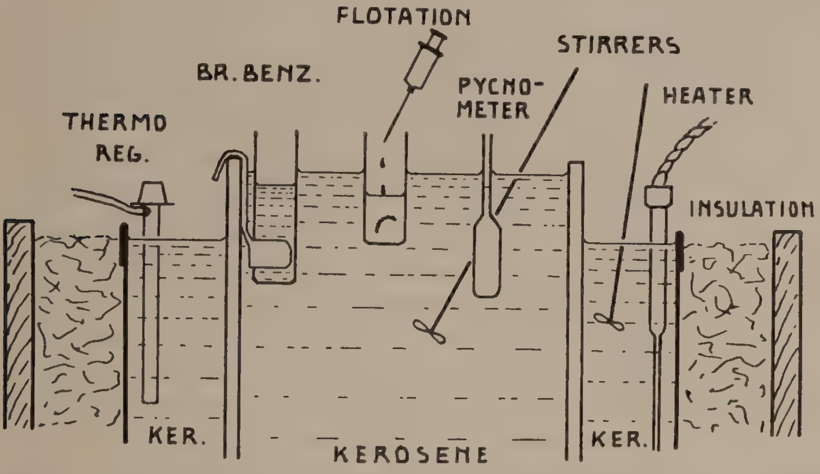

Fig. 1 Method for quantitative micro determination of ice in small gas-free objects by flotation.

The essential instrumentation will be seen from figure 1. It consists of an insulated double kerosene bath, which is accurately thermoregulated. To the edge of the central glass jar are attached a vial with bromobenzene, one holder for the 5 cc flotation vial, and one holder for a 0.7–1 cc pycnometer. One of these double baths is necessary for each desired temperature below freezing. For the above-freezing temperatures a water bath regulated to + 1° or + 20° may be used. We employed four of the double kerosene baths, which were placed in a cold room which was kept colder than the lowest temperature desired.

Procedure. The larva, thawed out of the ice, was placed on a filter paper and immediately refrozen, usually at — 20°. It was transferred by means of a fine glass hook to the coldest flotation vial, containing 1–2 cc kerosene taken from the inner bath with a syringe. Bromobenzene of the same temperature was added and thoroughly mixed by shaking until the larva remained suspended in the liquid, allowing ample time for temperature equilibration. With a dry syringe enough of this mixture was transferred to the pycnometer to fill it to the mark. When the meniscus remained constant at the mark, the temperature of the bath was read, and the pycnometer was taken to the laboratory, where it was warmed to room temperature by the hand to prevent moisture from condensing on it, and subsequently weighed. The larva was then transferred to the flotation vial of the next bath and the procedure was repeated.

The last flotation was performed in the laboratory at + 20°, or better on a cold porch at + 1°. The larva was finally taken out of the vial by means of a glass hook, quickly dried off on a filter paper, and weighed. In air the larva lost moisture at a rate of about 0.3 mg a minute. We have added 0.3 mg to all live weights to compensate for this weight loss. The larva was then dried overnight at 100° and the dry weight was taken.

Theory and calculations. When one part of water at 0° freezes to ice at 0°, the volume increases by 0.0907. The specific volume of ice at 0° is hence 1.0907. When ice is cooled it contracts according to a cubical coefficient of expansion of 153×10^{-6} (Dorsey, '40).

Let us assume that our object consists of W grams total water and D grams dry matter and that x grams of the water freeze to ice when the object is placed at — 1°. The volume of the dry matter we call y cc. We have then the specific volume at + 1°:

$$S_{+1} = \frac{W + y}{W + D}\text{, and hence}$$

$$y = S_{+1}(W + D) - W \qquad (1)$$

At — 1° the specific volume of the system is:

$$S_{-1} = \frac{1.0907x + (W - x) + y}{W + D}$$

Substituting for y and solving for x we get:

$$x = \frac{W + D}{0.0907}\,(S_{-1} - S_{+1}), \tag{2}$$

which, expressed in grams ice per 100 grams, equals

$$1103\,(S_{-1} - S_{+1}). \tag{3}$$

We notice that the volume of dry material, which remains essentially constant at this slight temperature change cancels out in the equations.

When operating through greater temperature intervals, corrections must be applied for thermal volume changes affecting (1) the pycnometer volume, (2) the ice volume, and (3) the water volume.[6] Applying these corrections to (3) and assuming for an example that the determinations were made at + 20° and − 20°, we have:

grams ice in 100 grams tissue =

$$1103\left(\frac{\mathrm{vol}_{-20}\,(1 - 40\,\gamma)}{\mathrm{weight}_{-20}\,(1 - 20\,\beta)} - \frac{\mathrm{vol}_{+20}}{\mathrm{weight}_{+20}\,(1 + \alpha)}\right) \tag{4}$$

where γ is the cubical thermal expansion coefficient of glass, β is the cubical expansion coefficient of ice, and α is the cubical expansion of water taken from + 20° to 0°.

As a check upon the method we have determined the amount of water frozen in small (about 10 mg) pieces of bubble-free clear ice. The determinations, made at − 20°, − 10°, and − 5°, were accurate to ± 2% (table 6).

It has long been recognized that it is very difficult to freeze a given quantity of water to ice without getting air bubbles formed. It requires a strict vacuum technique to succeed (cf. Bunsen, 1870; Dorsey, '40). Fresh water at 0° dissolves 2.9 volume per cent air, and if this were to come out as bubbles in the ice it would completely invalidate the flotation results. By experiments to be described (p. 40), it has been shown that the dissolved gases in ice, if any, are less than 0.001 of the gases contained at 0° in water saturated with air. In other words, unless the dissolved gas in the water can somehow escape it will be trapped in the ice as bubbles. This is the reason for the masses of bubbles in ice cubes frozen in the refrigerator. When lake ice forms the frozen-out gases can be taken up by the underlying water, and in this way bubble-free ice may be formed.

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[6] We have disregarded corrections for (a) the thermal contraction of the dry substance going from + 20° to − 35°, (b) the expansion of water going from − 1° to − 35°, and (c) the expansion by colloidal dehydration (0° to − 35°). In our larvae the volume of the dry matter is about 12% of the total volume. Taking a large coefficient of volume expansion, such as found in some plastics, of 3×10^{-4} our specific volume at − 30° might be off by about 1: 1000. This slight error is counterbalanced by the thermal expansion of the unfrozen water and by dehydration expansion of the colloids (cf. Svedberg, '24).

In the larvae we must assume that as freezing proceeds the dissolved gases are concentrated in the unfrozen liquids, building up the tension, and the question arises, do bubbles form?

TABLE 6

Percentage of frozen water in clear bubble-free ice (about 10 mg)

TEMPERATURE	−20°C.	−10°	−5°
	%	%	%
Experiment I	101	101	98
Experiment II	101	101	98

This can be decided by a very rapid thawing of a frozen larva in warm water under the binocular. If free gas were frozen out in the larva it would be visible as bubbles, since the larva turns transparent as it melts. Bubbles were never observed, whether the larva had been frozen in air, in kerosene, or by natural processes. Neither did any internal bubbles appear when the larva was immediately plunged into water in a tube and evacuated under water. That bubbles do not freeze out is a remarkable fact. At least some species of chironomids are known to acquire air in their tracheal systems in the latter part of the larval stage (Pause, '19). Observations under the microscope with or without evacuation proved that our larvae had no air-filled tracheae.

We may hence conclude that in this material the flotation method can be accurately applied. This is also well borne out by the results. The freezing out of gases in the commonly used dilatometric methods is a source of error, which has been largely overlooked.

We have also applied the flotation method to small pieces of a lichen. In this case the piece was first evacuated, while in the kerosene, so as to get rid of the entrapped air as completely as possible.

Results

In figure 2 we have plotted the relative amounts of ice and unfrozen water in relation to weight of dry substance at

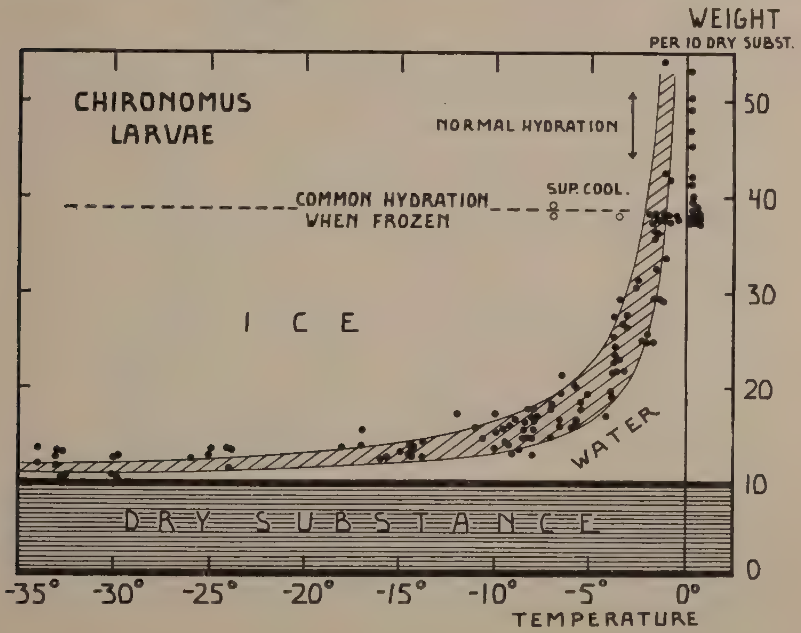

Fig. 2 The relation of unfrozen water and ice to the dry weight of *Chironomus* larvae at graded freezing temperatures. The shaded area is limited by two hyperbolic curves drawn so that the free water is inversely proportional to the temperature below freezing. The lower curve has a freezing point depression of 1° when the amount of free water is 30 parts; the upper curve has the same amount of free water when the freezing point depression is 2°. It will be seen that the empirical data are rather well described by assuming that the free water per dry substance is inversely proportional to the temperature below freezing, starting with a freezing point between — 1° and — 2°. The data are based on 26 individual curves.

graded temperatures below zero. The plot is based on 26 individual curves, each with three or four temperatures below freezing. The amount of unfrozen water decreases in a hyperbolic fashion with decreasing temperature, i.e., when plotted as concentration versus temperature the relation is nearly linear (fig. 3). As can be seen from figure 2 the amount of free water at each temperature varies actually by as much as 100%, or even more below — 25° to — 30°.

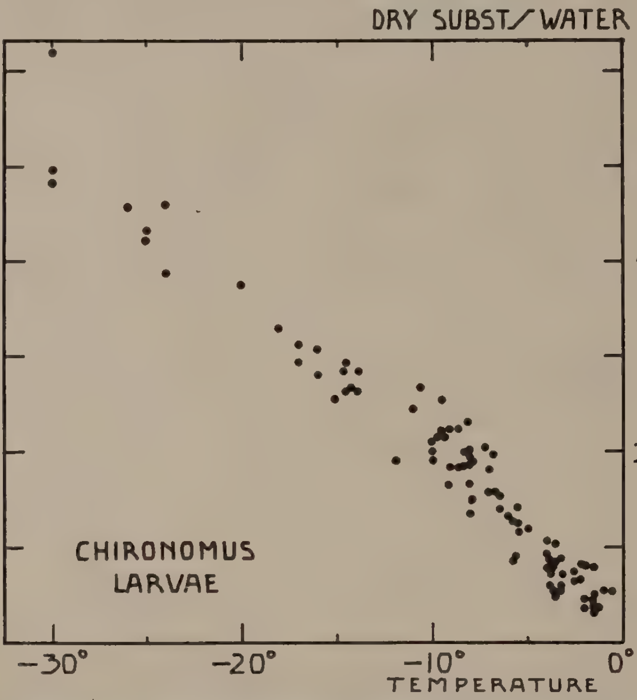

Fig. 3 The relation between temperature and concentration of dry matter in the unfrozen water of *Chironomus* larvae at graded temperatures below freezing. The 26 individual curves on which the data are based have been reduced to the same average slope by applying individual factors. The figure shows that there is an overall rather linear relation between the concentration of dry matter in free water and temperature.

The incipient freezing point of our larvae was near — 1° in the hydrated state, which corresponds to the average found in several insects (cf. Prosser, Bishop, Jahn and Wulff, '50). At the lower concentrations on the curve (fig. 3), it appears that the freezing point lowering is nearly proportional to the increase in concentration, as is well known for dilute solutions of crystalloids. At higher concentrations the curve bends down somewhat, i.e., the freezing point depression increases more than the concentration. This may happen at higher concentrations in crystalloids, like glucose, and may also be produced by the increasing concentration of colloids (cf. Crafts, Currier and Stocking, '49). Somewhat similar curves obtained by other methods have been presented by several authors, e.g., Ditman, Vogt and Smith ('42) on a variety of insects.

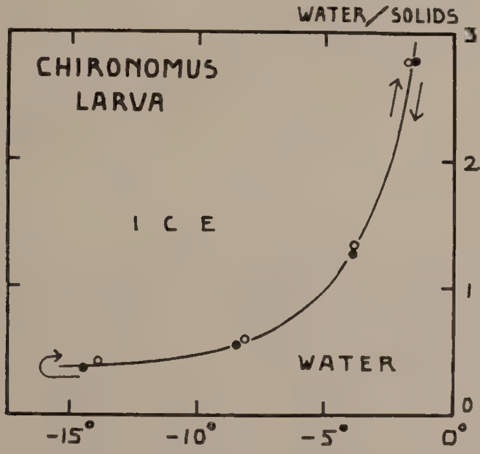

Fig. 4 Relation of ice to free water in a chironomid larva on descending and ascending temperature scales. The process is perfectly reversible.

In figure 4 one of four experiments is presented where ice was determined first at a descending temperature, then at an ascending temperature, and it is seen that the process is thoroughly reversible, with the same equilibrium between freezing point and free water whether the temperature is going down or up. This fact throws light upon the much-debated question of whether the unfrozen water is merely in a state of supercooling or not. At — 15° 90% of all water is frozen, and this quantity is therefore already seeded with ice crystals. If we presume simple supercooling, with no forces opposing the freezing, the curve could not possibly retrace itself but would run horizontal upon warming until the melting point was reached at some — 1°, when all the ice would melt. That we are here dealing with a true equilibrium and not with supercooling is

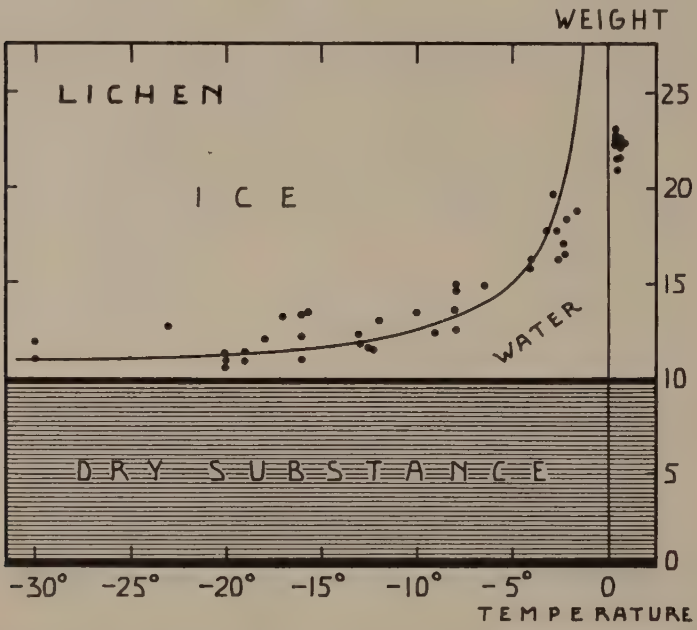

Fig. 5 Relation of ice to unfrozen water in a lichen *Cetraria richardsonii* at graded temperatures below freezing. The data compare fairly well to a hyperbola (full drawn line).

also brought out by the regularity of the freezing curve and by some experiments where the larva was exactly balanced in the bromobenzene-kerosene mixture at — 4°, i.e., on a steep grade of the curve. If now mashed against the bottom of the vial the larva still remained exactly balanced. If the unfrozen water were due to supercooling one would expect that at least some of it would freeze when the larva was mashed, but such was not the case.

Thirteen ice determinations were performed on three larvae which had been coagulated by boiling in water for one minute. In spite of the gross change in colloidal properties the results did not differ from those obtained with unboiled animals. Moran ('26, '35) found similarly reversible freezing curves in heat denatured egg albumen and in gelatine.

Supercooling was observed several times in our larvae in experiments where the flotation was determined at a descending temperature (fig. 2), but it is not a condition for survival in our chironomids. In these animals there is for each temperature a specific equilibrium ratio between the unfrozen water and the solids, so that the free water is approximately inversely proportional to the number of degrees below freezing.

A similar series of ice determinations at graded temperatures was performed on a lichen *Cetraria richardsonii* Hook. (fig. 5), using the flotation method. Due probably to the difficulty of getting rid of the air entrapped amongst the hyphae these results came out with a considerably greater spread than that shown by the larvae experiments, but they show the same general trend.

IV. RESPIRATION OF FROZEN ORGANISMS

It was pointed out in the previous chapter that many arctic, subarctic, and boreal plants regularly freeze during the winter, with easily demonstrable ice formation in their tissues. It was likewise shown by quantitative determinations that a chironomid larva and a lichen regularly have up to more than 90% of their total moisture frozen to ice during the coldest months of the winter.

We may wonder, with this large quantity of ice present in the organism and the severe dehydration of solutes and colloids which this produces, how the life processes are affected. It will be shown in chapter VI that ice may be regarded as possessing practically zero diffusibility for gases and practically zero solubility. The large amounts of ice in the living *Chironomus* may hence present a formidable lifeless diffusion barrier for the processes that might still go on associated with the little moisture that remains unfrozen. It is our task in this chapter to find out what, if any, respiratory metabolism might take place in such frozen plants and animals.

Several authors have tackled this problem. Jumelle (1890) measured the respiration of several lichens at low temperatures. He found no observable oxygen consumption in a naturally moist lichen, *Parmelia caperata,* during five hours' exposure to a temperature which varied between — 2° and — 9°C. Neither did he find any after 14 hours at the same temperatures in *Evernia (Physcia) prunastri,* nor was there any detectable assimilation. When moistened first, however, some gas exchange could be determined. In 1891 the same author reported measurable assimilation in twigs of spruce *(Picea excelsa)* and *Juniperus communis,* and in three species of lichens, which had been kept for a few hours at fluctuating temperatures, reaching as low as —32° to — 35°. In 1892 he stated he was unable to observe respiration below — 10° in five lichens *(Parmelia acetabulum, P. caperata, Physcia ciliaris, Cladonia rangiferina, Peltigera canina).* The experiment lasted only a few hours, and the temperature was allowed to fluctuate considerably.

Henrici ('21, tables 5, 6, 8) listed six species of lichens with CO_2 production at temperatures from + 24° to — 6° to — 16°, and gave similar data for a series of alpine vascular plants. The lichen data failed to reveal any consistent temperature influence on the respiration even at positive temperatures, and hence the technique employed must have been inadequate to analyze the respiration below freezing. The considerable inconsistencies in the phanerogam data point to the same conclusion.

Zacharowa ('29) determined the CO_2 production in the dark of pine twigs *(Pinus silvestris)* near Moscow from + 11° to — 14°, but found no correlation between temperature and respiration in this interval. Newton and Anderson ('31) recorded the respiration of overwintering wheat plants of different varieties, using the living parts without roots. They found the hardier varieties had less winter respiration than the not-so-hardy when run at — 7°. The Q_{10} calculated from the data shows an average of about 2.3 from — 7° to 0°, and 3.3 from 0° to + 7°.

Kalabuchov ('34) reported in *Tenebrio* larvae an oxygen consumption at — 7.5° to — 10° nine times less than that at + 15°. Kozhantchikov ('35, '38, '39) determined the oxygen consumption in a large series of insects by means of a modified Barcroft respirometer, where the chambers could be closed and removed from the manometer and could be separately held at the desired temperature. He reported that in the cold-hardy species there was virtually a "temperature-stable" respiration persisting below 0° to as low as investigated, namely, towards — 20°C. The method used is, unfortunately, open to a fatal objection in that the material had to be heated to 0° or above to make the reading, and hence two periods of highly accelerated respiration are included in the measurements (cf. p. 30).

It would seem from the above survey of data on respiration below freezing that little consistency has so far been attained. One reason for this is undoubtedly that the techniques in many cases have been inadequate to deal with the problem at hand.

Material

The respiration at graded temperatures was followed down to — 20° to — 26° in overwintering parts of the following five species of vascular plants: twigs of alder, *Alnus crispa* (Ait.) Pursh., and willow, *Salix alaxensis* Cov., from Umiat, and rhizomes, basal shoots, and roots of *Elymus arenarius* L., *Papaver radicatum* Rottb., and *Petasites frigidus* (L.) Fries, taken near the laboratory at Point Barrow. The following lichens, from the same vicinity, were also used: *Cetraria delisei* (Bory) Th. Fr., *C. islandica* (L) Ach., *Dactylina arctica* (Hook.) Nyl., *Thamnolia vermicularis* (Sw.) Ach.

In addition to these plants the respiration of the red chironomid larvae was followed down to — 15°. The plant material was dug out of the snow or frozen ground during February and

March, 1948. The larvae were obtained from frozen mud chopped out of the "Lepidurus" pond on April 26, when it was still full winter.

Method

Principle. The plants or larvae were enclosed in a glass chamber with a known amount of atmospheric air. After allowing ample time for equilibration at a temperature at or below the desired temperature the material was evacuated and fresh outdoor air let in. The chamber was sealed and left thermoregulated at the desired temperature until the respiratory exchange could be adequately analyzed by gas analysis.

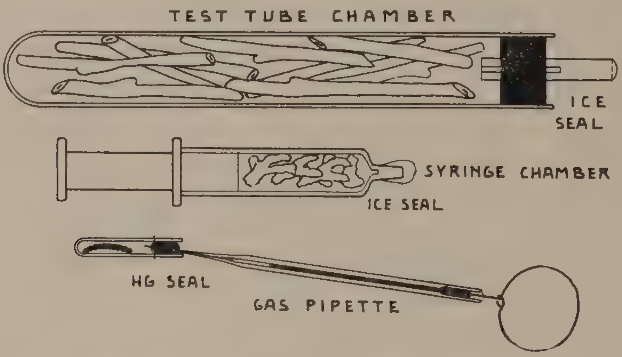

Fig. 6 Different chambers used for gas analytical determinations of respiration below freezing.

Procedure. The plant material was cleaned and prepared at around 0°. No provision was made to seal off the cut ends of twigs or lichens, as we were mainly interested in the relative effect of the temperature. The twigs or lichens were placed either in syringe chambers or in test-tube chambers. The syringe plunger was lubricated with glycerine and the thick-walled test tubes were provided with glycerine-lubricated Neoprene stoppers pierced by capillary glass tubing (fig. 6).

The loaded tubes or syringes were left for several hours outdoors usually at — 20° to — 25°, well below the desired temperature. The air was then renewed with outdoor air by pumping the syringes or by three times' evacuation of the tubes, taking care never to heat the chambers. In order to seal the chambers they were held in cold tongs or heavy mittens. A drop of water was placed in a depression in a heated rubber stopper. The capillary or nozzle was pressed into this drop and the water would freeze to ice and form a seal in a few seconds, whereupon the chambers would be put in a dark thermoregulated Prestone bath, where they might remain for as long as three and a half months.

Gas samples were obtained by attaching a conventional mercury gas pipette to the capillary or nozzle of the respiration chamber by means of a chilled piece of pressure tubing. The stopcock of the gas pipette was filled with mercury, and by lowering the levelling bulb gas tightness of the ice seal was ascertained. Gas connection was established by applying a bare finger to the rubber tubing which would melt the ice in the capillary. The whole procedure was performed at a temperature colder than that of the bath. The tubes were now prepared for the next desired temperature.

The gas sample was analyzed with the $\frac{1}{2}$ cc analyzer (Scholander, '47) to an accuracy of $\pm 0.01\%$ of the CO_2 content and $\pm 0.015\%$ of the oxygen content (cf. Hock, Erikson, Flagg, Scholander and Irving, '52). At 0°, $-5°$, $-10°$, $-15°$, and $-26°$, the plants were left in the chambers for the following approximate times: 6, 20, 55, 240, and 1880 hours. The approximate average ΔCO_2 (and ΔO_2) percentages obtained were then 3, 3, 0.5–1.5, 0.4–1.5, 0.06–0.14. At $-26°$ the data are obviously not very accurate.

The total gas content of the tube containing the respiratory material was determined by connecting the evacuated tube to a syringe with its plunger pulled out. On opening the connection between them gas would fill the tube from the syringe, and the volume would be read directly on the syringe. From the changes in gas composition and the total air volume the CO_2 production and O_2 consumption could be calculated, when proper corrections for temperature and pressure were applied. As samples were only taken at the end of the runs we do not know to what extent the rate during the run remained constant.

The method depended upon the inertness and the diffusion tightness of the Neoprene stoppers and syringes. As to possible oxidation of the Neoprene, this would be a source of error only if it amounted to a detectable fraction of the respiration of the relatively very large amount of the living matter. No trace of oxidation of the Neoprene stoppers could be detected after 244 hours at — 15°.[7] The ice seal in the capillary was checked for gas tightness by vacuum testing at the end of each run. Leaks occurred in less than 2% of the runs. Diffusion tightness of the system was tested by leaving the chambers filled with an accurately known gas mixture. Using relatively very high CO_2 concentrations (4%) and correspondingly low O_2 concentrations, neither test tube chambers nor syringes showed detectable diffusion loss.

The chironomid larvae were run singly in eight narrow snugly fitting vials of about 40 mm^3 capacity (fig. 6). Each tube was calibrated to a mark. The tubes were kept horizontal in holes drilled in a piece of wood which served as a stand. The larvae were flushed out of the frozen mud and were transferred one into each water-filled tube. The water was removed by suction, and the stand with the tubes was placed outdoors at a temperature lower than the desired temperature for several hours. A fine suction tip was introduced into the tubes to renew possible stagnant air, and a drop of cold mercury was deposited as a seal in each tube opening, closing it at the mark. The tubes were placed at the proper temperature, where they would remain until enough change had occurred in the gas composition to allow an adequate analysis. The gas sample, of a few cubic millimeters, was drawn through the mercury seal (fig. 6) and analyzed as described by Scholander and Evans ('47) with an accuracy of ± 0.15% for the CO_2 and O_2. The mercury plug was removed and the block with larvae was prepared for the next desired temperature.

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[7] A slight blank value of 0.04–0.05% oxygen decrease was found in two tubes after 1920 hours at — 26°. If this correction were applied to the data at this temperature in figures 7 and 8 the values would be approximately halved. This would seemingly improve the curves, but would not materially change the picture.

Results

In figure 7 we have plotted the respiration of winter twigs of the older, *Alnus crispa,* and of the willow, *Salix alaxensis.* Above freezing the volumetric syringe respirometers were used (Scholander, '50) and below freezing the test-tube chambers and gas analysis. It will be seen that at zero the two methods overlapped and that below freezing the oxygen consumption dropped steeply. Essentially identical results were obtained in four species of lichen, *Dactylina arctica, Thamnolia vermicularis, Cetraria delisei,* and *C. islandica,* two of which are given in figure 8. In an earlier series, syringe chambers were used for roots and basal parts of three species of vascular plants, *Elymus arenarius, Papaver radicatum,* and *Petasites frigidus*

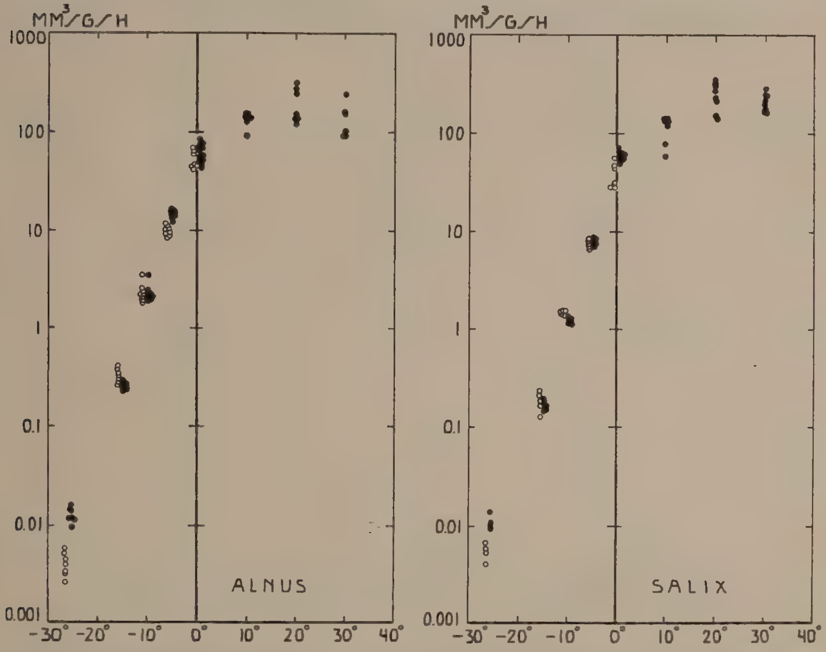

Fig. 7 Respiratory exchange in alder *(Alnus crispa)* and willow *(Salix alaxensis)* from + 30° to — 26°. Solid circles: Oxygen consumption. Open circles: Carbon dioxide production. Four to 8 separate determinations were made at each temperature.

(fig. 9). Again the result was the same, although with a somewhat larger spread of data. The micro determinations on the chironomid larvae also showed the precipitous but perfectly regular drop in respiration below freezing (fig. 10). Here data above freezing were obtained also on individual larvae using a volumetric microrespirometer (Scholander, '42).

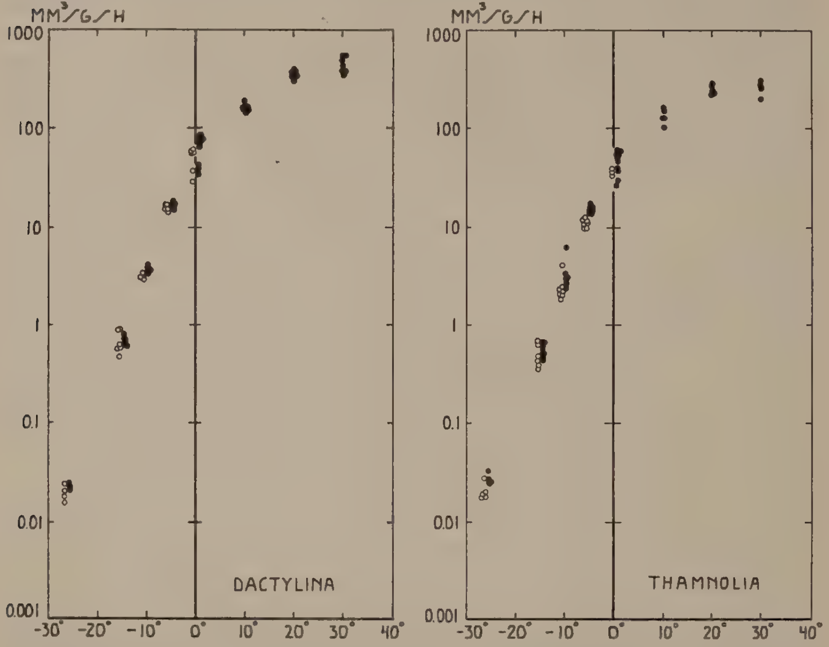

Fig. 8 Respiratory exchange in two lichens, *Dactylina arctica* and *Thamnolia vermicularis* from + 30° to — 26°. Solid circles: Oxygen consumption. Open circles: Carbon dioxide production.

In figure 11 we have plotted the change in respiratory rate as a function of temperature (Q_{10}) for the latest and presumably most reliable runs. Between zero and — 5° there is a precipitous drop in respiratory rate from a Q_{10} between 2 and 4 to a Q_{10} between 20 and 50.[8] If the survival time of an isolated organism depends upon the rate at which its energy stores are used up, then there will be an inverse relation between its survival time and metabolic rate. On this basis, at a Q_{10} of 50 an organism capable of surviving a modest ten days at 0° would last for a thousand years at — 23° and for a million years at — 42°! To what extent such mathematical extrapolations reflect biological reality is certainly not known.

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[8] Considering this extraordinary temperature sensitivity in the respiration below freezing it is clear that any technique that depends upon thawing of the material for a determination is doomed.

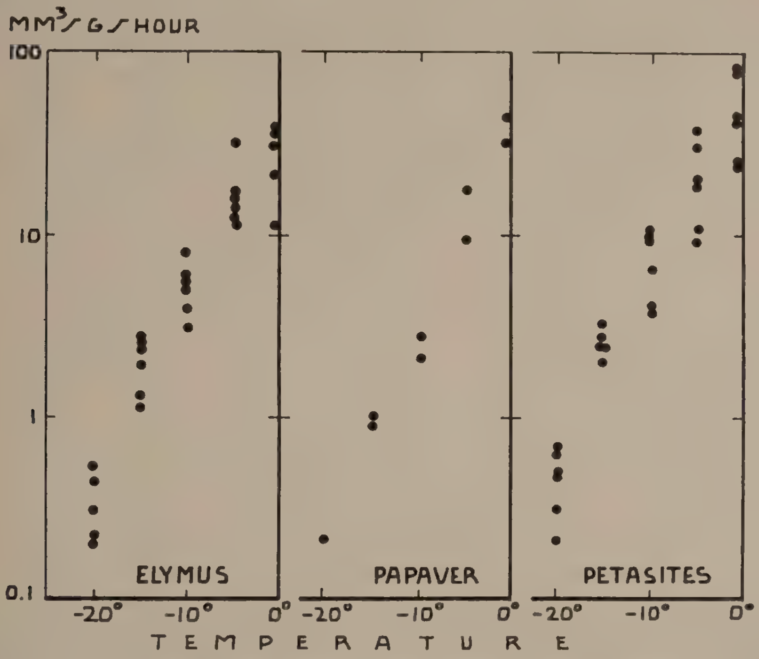

Fig. 9 Carbon dioxide production in three vascular plants *(Elymus arenarius, Papaver radicatum,* and *Petasites frigidus)* from 0° to — 20°, run in syringe chambers.

There can be no doubt that the very high Q_{10} below freezing is primarily caused by the ice formation. Even at — 5° a chironomid larva has some 70% of its water frozen; at — 10° 85% of the water is frozen and only 15% remains available as a vehicle for metabolic processes.

If supercooling takes place we would expect that the Q_{10} in the supercooling range would essentially extrapolate from the values above zero, which are in general somewhere around two to 5 (Scholander, Flagg, Walters and Irving, '52, '53). Newton and Anderson ('31) found a Q_{10} of about 2.3 in a cold-hardy wheat between 0° and — 7°, which may mean that they were dealing with supercooled rather than frozen plants.

The precipitous drop in respiration rate below freezing in our forms could be explained as the resultant of several factors, such as, (1) the straight temperature effect upon the respiratory rate, (2) the effect of the concentration of tissue fluid upon the rate, and (3) the effect of the ice on the gas diffusion. The last factor may be relatively unimportant, because of the fact that gas bubbles do not form in the chironomid larva upon freezing. We take this as an indication that tension equilibration to the outside must take place rather rapidly through the frozen larva, which again probably means that the

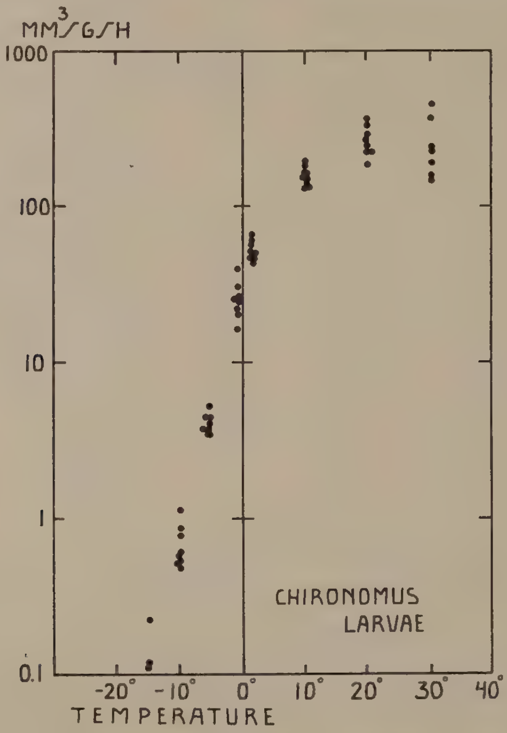

Fig. 10 Oxygen consumption in individual chironomid larvae from + 30° to — 15°.

fluid water is evenly distributed like a meshwork between minute ice crystals. This interpretation is supported by the fact that the larvae maintain a pasty consistency even at — 30°. Hence it seems likely that a very slow respiratory gas exchange can proceed through the frozen tissues with only relatively small tension gradients.

Within the temperature limits (— 5° to — 25°) of actual freezing, where the quantitative data on ice formation and respiration are relatively good, there is a rather striking constancy of the Q_{10}, and at the same time a rather linear relation between concentration of the dry matter in the free water and the temperature below freezing. This again may be taken to mean that there should be an exponential relation between the degree of dehydration and the respiratory rate. Numerically in the freezing range the respiratory rate of our chironomid larva decreases some 40 to 50 times for a difference of two units in the ratio of dry weight to water.

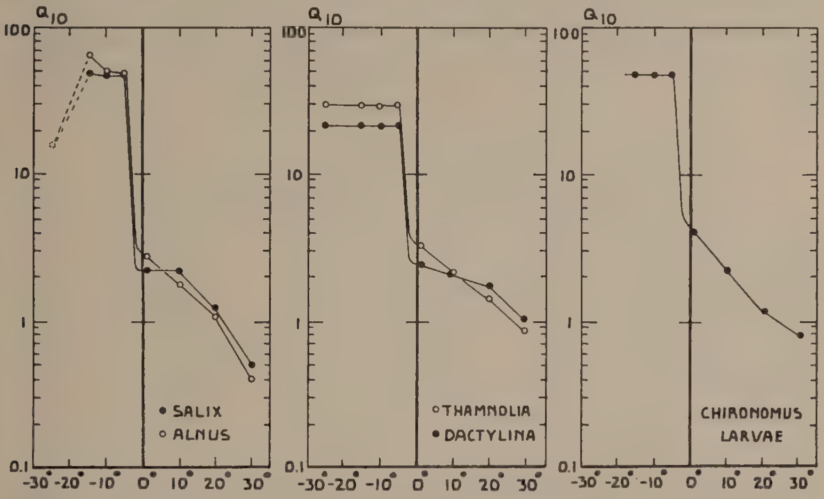

Fig. 11 Change in respiratory rate with temperature expressed as Q_{10} in willows, alder, two species of lichens, and chironomid larvae.

There are many studies of the effect of dehydration on respiration at normal temperatures, but very few are concerned with such high degrees of dehydration as we are dealing with here. When the dehydration becomes excessive, as in many seeds, resting spores, and some eggs, the metabolic rate may have plunged down to almost imperceptible values, as evidenced by the fact that the organism may remain dormant in this state for several years.

Several authors (Jumelle, 1892; Fraymouth, '28; Cuthbert, '34; Smyth, '34) report a near linear relation between the rate of respiration and the moisture content of lichens, whereas there are strong indications that when grains lose moisture the respiration decreases much more steeply. Air-dried *Acacia* seeds increased their respiration 10,000 times when moistened (White, '09), and in other desiccated seeds respiration could not be detected at all (Miller, '38). Desiccated eggs of some Collembolas and grasshoppers may remain viable for months and years (Wigglesworth, '39), and presumably therefore they are running at an extremely low metabolic rate. Accurate respiratory measurements at low moisture content may be technically difficult to obtain, but they must be made before we can properly judge as to the relative importance of the separate factors which bring about the high Q_{10} in our experiments, namely, the straight chemical Q_{10} of the unfrozen material, the diffusion hindrance by the ice, and the dehydration.

TABLE 7

Apparent respiratory quotient, RQ, below freezing

(Each figure represents average of 6–8 determinations; spread about ± 0.1)

TEMPERATURE	0°C.	−5°	−10°	−15°
Willow *(Salix alaxensis)*	0.65	0.92	1.20	1.50
Alder *(Alnus crispa)*	0.78	0.84	1.05	1.30
Thamnolia vermicularis	0.78	0.72	0.76	0.90
Dactylina arctica	0.79	0.83	0.81	0.80
Cetraria delisei	0.70	0.82	0.83	0.80
Chironomus larva	0.2–(0.8)	0.8	1.1–2.1	0.9–1.6

During our respiratory studies both oxygen consumption and CO_2 production were taken (figs. 7 and 8). In table 7 we have summarized the average apparent respiratory quotients obtained at different temperatures. In the willow and alder the CO_2 production immediately below freezing was relatively low, and at lower temperatures it increased. This is even more marked in the larva. It is believed that the irregularities in the CO_2 may be due to such factors as liberation of CO_2 from tissue fluids by freezing and/or absorption of CO_2 in the fluids when the tension builds up in the chamber (cf. Denny, '47).

It is of some interest to note that we hardly observed a single day, no matter how cold, when the ice would not melt if it were exposed to the direct rays of the sun in such favorable places as the south faces of rocks or bark of trees. In these places plants must endure being thawed and refrozen innumerable times, with consequent enormous changes in the cellular hydration and the metabolic rate.

V. EFFECTS OF FREEZING ARCTIC PLANTS AT LIQUID OXYGEN TEMPERATURE

We saw in the previous chapter that the respiration below the temperature of incipient freezing of plants and insect larvae dropped precipitously in all material so far investigated by us, but was still measurable at — 26°. Around Fairbanks and other places in the northern forested interior of Alaska and northern Canada some of the lowest known winter temperatures have been recorded (table 4). Overwintering buds and twigs and many other exposed structures are chilled sometimes down to — 60°, and one might wonder whether there is any limit to their cold hardiness. Many temperate forms of plants and animals have been exposed to liquid air temperatures, but only those forms that can stand a high degree of dehydration have been found able to survive (see survey in Luyet and Gehenio, '40, table 2). Experiments on plants from places with extreme winter temperature do not seem to have been made.

In the middle of March, 1948, twigs from the willow, *Salix alaxensis,* and the alder, *Alnus crispa,* were collected at Umiat in the northern foothills of the Brooks Range and were taken to Fairbanks, together with lichen material from Barrow. At Fairbanks twigs were collected from spruce *(Picea glauca),* larch *(Larix laricina)*, birch *(Betula* sp.), and cottonwood *(Populus tremuloides)*. The material was taken to the Alaska Chemical Company, where the Manager, Mr. Walter E. Buchow, generously placed the facilities of the plant at our disposal and supplied us with much technical help.

The frozen material was placed in large test tubes closed with a cotton plug. The tubes were immersed in Thermos bottles containing liquid oxygen at a temperature of — 183°C., where they remained for 18 hours. After our return to the Barrow laboratory, parts of the cold-treated and still frozen material were run through respiration tests at + 20°, together with controls, and another lot of the twigs, with controls, was planted in moist sand to test the capacity for development.

TABLE 8

Effect of exposure to — 183°C. on oxygen consumption at 20°C.

	HOURS OF EXPOSURE	RATE OF EXPOSED MATERIAL	RATE OF CONTROLS	RATE OF EXPOSED IN % OF CONTROLS
		mm³/g/hour	*mm³/g/hour*	
Lichens				
Cetraria delisei	18	186	277	67
C. richardsonii	18	287	300	96
Dactylina arctica	18	102	349	29
Thamnolia vermicularis	18	111	331	34
Bushes, trees				
Willow *(S. alaxensis)*	18	210	250	84
Alder *(A. crispa)*	18	210	200	105
Birch *(Betula sp.)*	24	71	15 [1]	470
Spruce *(P. glauca)*	24	52	61	85
Larch *(L. laricina)*	24	258	197	130
Cottonwood *(P. tremuloides)*	24	113	21 [1]	540

[1] These very low values are due to the fact that at this temperature etheric gases were produced by the buds to such an extent that the volume sometimes increased rather than decreased. On opening the respirometer the laboratory was filled with the exquisite spring odors of bursting buds.

The results of the respiration test are seen in table 8. All forms chilled to liquid oxygen temperature respired after the treatment, but many showed very considerable deviations from the controls. The reason for this soon became apparent from the behavior of the twigs planted in wet sand. All the control branches except the larch produced leaves in a week's time, whereas none of the liquid-oxygen-treated plants showed any sign of development. As organisms therefore these plants had all been killed by the liquid oxygen temperature, whereas respiration was still going on in the damaged tissues. The lichens had a normal appearance after the exposure, but whether as organisms they stood the treatment or not we cannot say, since growth would be extremely difficult to ascertain in a lichen (cf. p. 7). As to the reason for the death of the twigs of trees and bushes, we may only guess. It seems possible that in general the lowest limit of temperature which can be tolerated is that at which intracellular freezing of vital cells starts. It would seem that not even arctic material can stand liquid air temperatures unless the cells can tolerate at the same time a high degree of dehydration. Whether or not these organisms can stand a more or less complete intracellular ice formation is, however, not known.

VI. ICE AS A HABITAT FOR HIBERNATING ORGANISMS

A great many aquatic plants and animals spend the winter frozen into the ice of lakes and pools in arctic or other cold regions. Around Point Barrow shallow ponds are frequently more or less filled up with aquatic grasses, *Arctophila fulva* and *Dupontia fisheri,* and often mosses, all of which in a green, living condition freeze into the lake ice during the winter. Towards the surface there are often bubbles, frozen into the ice. If blocks are chopped out of this ice and clear vertical sections an inch or two thick are sliced off, frozen animal life can be observed in the ice. Numerous red copepods may be seen adhering to the grass straws and once in a while a red chironomid. In the bottom part, amongst the mosses, there may be purple eggs of *Lepidurus,* red eggs of copepods, and green and red species of chironomids in their plant debris tubes. Upon melting, all of these forms come to life within a few hours, along with many more species, such as *Daphnia*.

We know from chapter IV that respiration goes on at a measurable rate to at least — 26°. At the temperature where the organism is completely thawed, somewhere around — 1°, the natural metabolic rate is very considerable, and yet at that temperature the animal or plant may still be trapped within the lake ice. The organism may actually be free inside a little water hollow in the ice, which has been melted by body excretions and absorption of sunlight. One might ask what possibility there would be for such a form, enclosed by the ice, to maintain respiration. In other words, what are the solubility and diffusion conditions for gases in ice? Can respiration take place through ice?

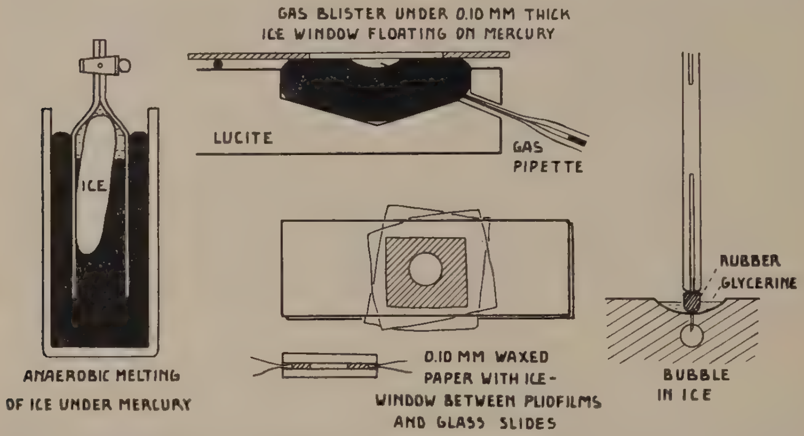

Fig. 12 Methods for determination of gases dissolved in ice, for determination of gas diffusion through ice, and for analysis of gas bubbles trapped in lake ice.

A clue to this problem can be found by analyzing some of the gas bubbles frozen into the ice. If the ice is permeable to gases, then bubbles near the surface should in time come into equilibrium with the air by diffusion through the ice, and towards the spring the bubbles should contain more or less air. Many analyses were made to test this situation.

With a carpenter's chisel the ice was shaved thin over the bubble to form a small depression. The depression was filled with a few drops of glycerine or concentrated acid citrate solution, and the gas pipette, provided with a needle and a small rubber gasket (fig. 12) was inserted anaerobically into the bubble. Enough gas for analysis would usually enter the capillary by over-pressure in the bubble. The gas could then be analyzed in a capillary syringe analyzer (cf. Scholander, Claff, Teng and Walters, '51). Duplicates would usually agree within 0.4%. As absorbents KOH and alkaline pyrogallol were used, and the unabsorbed remainder would contain nitrogen and probably more or less methane and other non-absorbable gases from decay.

TABLE 9

Composition of gas bubbles in ice from "Lepidurus Pond," which contained overwintering plants and animals, March 13, 1948

Accuracy ± 0.4%

DEPTH BELOW SURFACE	PER CENT CO_2	PER CENT O_2	PER CENT UNABSORBED
cm			
2	26.8	1.9	73.2
3	44.1	0	65.9
3	24.1	0	75.9
3	46.1	0	53.9
4	15.7	0	84.3
10	13.7	0	86.3
20	37.0	0	63.0

Table 9 gives the gas composition of bubbles in the ice of a small pond which contained mosses and aquatic grasses, as well as animal life. These data indicate that anaerobic conditions prevailed in the water at the time when these bubbles originally collected under the ice to be gradually enveloped as it thickened. They clearly show that, if any, the gas exchange through ice must be extremely slow.

It was noted that copepods, in particular, were frozen into the ice in great clusters next to the hollow internodes of straws and runners of the aquatic grass, *Arctophila fulva*, which projected through the ice surface. The gas composition in 13 of these internodes, taken mostly 1–3 dm below the ice surface, was analyzed. In all cases but three these showed atmospheric air. In the three exceptions there was 1 to 3% CO_2 and correspondingly lower oxygen. Whenever these roots remain gas filled, changes in barometric pressure and temperature, as well as convection by the inverse temperature gradient downwards, evidently keep them well ventilated.

Because of the importance of ice as a habitat for hibernating aquatic plants and insects we have endeavored to estimate directly the solubility of air in ice and the diffusion constant of CO_2 and oxygen through ice.

1. Estimation of solubility of air in ice[9]

A piece of bubble-free, clear, commercially frozen ice was sawed into a rod about 1 × 1 × 4 inches. The rod was shaved cylindrical with a knife, smoothed by the bare hand, and introduced under an inverted cylinder filled with mercury (fig. 12). When the ice started to melt, water and accidental bubbles which collected under the stopcock were discharged. After wasting about half of the melt water, the latter part was taken anaerobically into a syringe and from there 5 cc were transferred into a several times evacuated manometric Van Slyke apparatus. A minute gas bubble appeared after the extraction, too small to be recorded on the manometer at a 500 mm^3 volume. Hence the bubble would at most be some 0.15 mm^3. Five cubic centimeters fresh water at 0° would hold dissolved near to 146 mm^3 air. If our bubble originated from air dissolved in the ice, ice could at most hold 0.15/146, or a thousand times less gas than the water could hold. Three separate determinations were made, all with the same result. Considering that, even after repeated evacuations and ejections, a small gas bubble is usually left in the Van Slyke apparatus, it is likely that the solubility of air in ice is considerably less than a thousandth of its solubility in water.

2. Estimation of diffusion of gases through ice

Principle. A thin sheet of ice is floated on a pool of mercury. A known volume of 20% CO_2 and 80% N_2 is deposited as a blister under the ice, forming a circular contact area, which is measured. After 24 hours a sample of the gas is withdrawn and analyzed for change in composition. The diffusion is calculated from the ice thickness, area of gas-ice contact, gas volume, and change in composition.

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[9] Performed at the Department of Biological Chemistry, Harvard Medical School ('50).

Method. A $\frac{1}{4}$-inch hole is punched through a piece of paper 0.1 mm thick. The paper is lightly paraffined around the hole and cut out as a square. The thickness is measured on a micrometer. This square is sandwiched as a spacer between two sheets of thin Pliofilm and two microscope slides (fig. 12). Water is boiled and taken anaerobically into a syringe, which is provided with a fine hypodermic needle. The spacer hole is rapidly flooded with water from the syringe and the two slides are clamped together. Air bubbles must be avoided. The assembly is left to freeze in the cold room. When frozen and ready for use it is taken apart. The Pliofilm which prevents the ice from sticking to the glass is removed. We now have a circular piece of ice, 0.10 mm thick, held like a window in the paraffined paper frame. The ice must not be cracked. Very minute air bubbles seem impossible to avoid, as the filling of water into the frame is not anaerobic.

A piece of Lucite has been drilled half-way through with a $\frac{1}{2}$-inch drill. This cavity has a side channel (fig. 12). Mercury is filled into the cavity until it projects a millimeter above the surface of the plastic. When the assembly has attained the temperature of the cold room (— 20°) the paper frame with the ice window is placed on top of the clean mercury surface. Moving the ice back and forth on the mercury surface a completely dust-free and full contact is obtained between the ice and the mercury. A known volume of an accurately known gas mixture of near 20% CO_2 and 80% N_2 is delivered through the mercury up under the ice where it forms a circular contact with the ice. The blister is quite shallow and gives a favorable ratio between the area and the gas volume, without which the determination cannot be made. The gas is delivered through a calibrated pipette (fig. 12). Ours delivered 7.6 mm^3 at 20°. Two diameters are measured of the air blister and the area is calculated. The assembly is surrounded by snow or cracked ice and covered with a beaker to keep evaporation as low as possible. After a known time, e.g., 24 hours, a few cubic millimeters of the gas are withdrawn for analysis (Scholander and Evans, '47).

Results. The data of three determinations are given in table 10. Assuming no exchange of nitrogen through the ice because of equal tension on both sides, the diffusion of CO_2 and O_2 can be estimated and recalculated to desired units, for instance, as cubic millimeters of gas, 0°, 760 mm, going through one square centimeter of ice, 1 mm thick, with 1 atm gas pressure difference on both sides, and per hour. The micro gas analysis, including transfer, is accurate to ± 0.15%. The diffusion constant for CO_2 through ice at $-20°$ came out 0.006 to 0.01 and for oxygen less than 0.002 mm³/cm²/mm/atm/hour.

TABLE 10

CO_2 and O_2 diffusion through ice

Temperature, -20°C. Gas at start: 19.68% CO_2, 0.48% O_2, 79.84% N_2. Thickness of ice, 0.10 mm. Diffusion constant expressed as mm³/cm²/mm/hour/atm

EXP.	HOURS	BUBBLE AREA	BUBBLE VOL.		Δ %	Δ *mm*³	DIFFUSION CONSTANT
no.		*mm*²	*mm*³				
1	23.7	13.8	6.4	CO_2	-0.7	-0.045	0.007
				O_2	$< +0.2$	$< +0.013$	< 0.002
2	25.0	12.6	6.4	CO_2	-1.0	-0.064	0.01
				O_2	$+1.0$	$+0.064$	0.01
3	23.0	13.8	6.4	CO_2	-0.6	-0.039	0.006
				O_2	$< +0.2$	$< +0.012$	< 0.002

The diffusion constant for oxygen through 20° water was determined by Hüfner (1897), and recalculated by Krogh ('19) as 0.34 cc/0.001 mm/cm²/min/atm, or 20.4 in the units of table 10. The diffusion constant for CO_2 in water is some 20 times higher (Krogh, '19), or about 410 at 20°. Comparing these to our figures for ice of 0.006 to 0.01, we see that CO_2 diffuses some 40,000 to 70,000 times slower in ice than in water. During the experiment some thinning of the ice through evaporation loss was unavoidable, which makes our diffusion figures rather too high than too low. They may also be high due to the presence of very fine air bubbles in the ice, visible by magnification. We do not know to what extent diffusion could possibly have taken place through the thick mercury seal rather than through the ice. Neither do we know to what extent an ice surface is capable of adsorbing gases, and there might be a possibility of some differential ''creep'' of gas at the ice mercury interface. Needless to say, confirmation of our figures, especially at different temperatures, is much to be desired.

As a comparison to our figure for diffusion through ice of 0.006–0.01 we may give the figure for the diffusion of helium through silica at room temperature as 0.11, whereas air through silica diffuses more than 10,000 times slower again (Rayleigh, '36).

It should be noted that gas diffusion through ice, although evidently not before investigated, is not incompatible with the ideas of Quincke ('05) on the structure of ice. He considered that impurities might concentrate on the ice crystal surface as separating films of low melting point. The existence of such layers cannot be doubted (Dorsey, '40). This concept leaves open the possibility that dissolved gas may penetrate through these inter crystal films and may explain why the large CO_2 molecules can penetrate faster than the smaller O_2 molecules. If this is the explanation for the gas diffusion through ice it can be anticipated that the permeability will increase with increasing temperature. This was not tested. It is not unlikely that the presence of an organism might substantially improve the diffusion. We quote from a letter received from Dr. N. E. Dorsey: ''It seems to me not impossible that the gases and excreta might under suitable conditions not only increase the concentration of the films, but might even produce and maintain minute tubes through the developing ice mantle.'' Copepods were often found clustered around the air-filled grass stems frozen into the ice. The relatively aerobic conditions prevailing around these stems may well be of some importance for the animals' survival.

Using what is probably a high figure for oxygen diffusion through — 20° ice of 0.001, it is possible to estimate how much oxygen could at best diffuse to a larva rolled together to a sphere of 2 mm radius and situated in the center of an ice sphere with 1 dm radius at — 20°. According to the diffusion formula given by Barrer ('41, [26]) for such a situation the figure would come out to about 0.0025 mm^3/larva/hour/0.2 atm. A 20 mg larva at — 15° uses some 0.002 mm^3/hour. Below — 15° it seems therefore that the diffusion through the ice might keep pace with the extremely slow respiration. At least CO_2 need not seriously accumulate. If, as is usually the case, the larva is situated deep beneath a horizontal ice surface, and at a higher temperature, the situation probably gets very near to anaerobic.

As seen from table 9 it was repeatedly found that gas bubbles in pond ice 3 cm below the surface and deeper, and probably shallower as well, had no detectable oxygen in ice about 5 months (= 3600 hours) old. Let us assume a 5-mm-deep, large, flat, horizontal bubble chamber, situated 3 cm below the ice surface and filled with oxygen-free gas, somewhat like a dense mass of gas bubbles of the diameter usually analyzed. If oxygen diffused perpendicularly through the ice according to a diffusion constant of 0.001, we may calculate that 0.024 mm^3 oxygen would enter the chamber per square centimeter in 5 months. This would be diluted into 500 mm^3 gas per square centimeter and would give an O_2 concentration of 0.005%, or around 100 times less than could be detected by our gas analysis. There is hence no contradiction between the apparently oxygen-free bubbles and the estimated rate of oxygen diffusion. It is also clear that the CO_2 would hardly perceptibly be able to leave the bubble during the winter.

As far as chironomids go, this possibility of a very slight gas exchange through the ice is probably of little importance, since these animals are usually frozen in the bottom mud, which even thawed and without an ice cover may be more or less completely anaerobic. During the probably rather long period when the ice is melting it remains near to 0°. The larvae are then thawed and will tend to run at a high metabolic rate. Relative to this rate the gas exchange through the ice must be entirely negligible. It is well known that many chironomids can endure prolonged complete anoxia in their natural habitat as well as in the laboratory. At 0° to 5° Lindeman ('42) found that some species of chironomids survived four months when kept in completely anaerobic bottom ooze.

3. Mechanical effects of freezing in ice

Besides the fact that ice must be regarded as practically gas-tight, there are mechanical factors in the process of the freezing of water which strongly limit ice as a normal hibernating medium. Thus we were never able in the laboratory to freeze the large red chironomid larva *in ice* without killing it, by rupturing its skin. *Lepidurus* was likewise mechanically damaged and killed by freezing in ice. Copepods, when frozen in a drop of water held in a small wire loop, would most often be killed, ruptured by an air bubble, and on thawing discharged a cloud of oil drops. Only in a few cases did we succeed in getting the copepods and a small greenish chironomid through the experimental freezing in a water drop at — 15° without visible damage. After thawing the copepods remained for some time immotile, and then would suddenly start full activity. There were thousands of these copepods frozen naturally in the ice, as could be seen through clear sawed-out sections. When thawed out the majority of them would survive, but some would be killed by internal gas bubbles.

A principal difficulty in experimentally freezing animals in water is that the gases are trapped in the ice and form bubbles which rupture the tissues. When frozen out of water on a piece of filter paper, however, the copepods would almost always survive. Freezing and thawing of an organism and its excreta inside ice must also give rise to considerable positive or negative pressures, which would cause damage, especially in the presence of free gases.

VII. EXPERIMENTS ON FREEZING THE BLACKFISH *(DALLIA PECTORALIS)*

A visitor to Alaska who is interested in animals will soon hear of the wonders of the blackfish which freeze solid in the winter, of ice worms living in the glaciers, and of the even more remarkable ice snakes!

The blackfish occur commonly in the Yukon and Kuskokwim water basins, where they are caught in great quantities in fish traps set through the ice. Their remarkable abilities have been colorfully described by Turner (1886). The fish are put in big baskets. "Here the fish are exposed to the severe temperature and cold winds. The mass of fish in each basket is frozen in a few minutes; and when required to take them out they have to be chopped out with an ax or beaten with a club to divide them into pieces of sufficient size to be fed to the dogs, or put into the pot to boil.

"The vitality of these fish is astonishing. They will remain in those grass-baskets for weeks, and when brought into the house and thawed out they will be as lively as ever. The pieces which are thrown to the ravenous dogs are eagerly swallowed; the animal heat of the dog's stomach thaws the fish out, whereupon its movements soon cause the dog to vomit it up alive. This I have *seen,* but have heard some even more wonderful stories of this fish."

This observation on the revival of frozen fish is not entirely unique, for according to W. H. Martin ('15) fishermen of the Bay of Fundy say that in very cold weather a herring, apparently frozen stiff when thrown on the ground, will swim off when thrown back into the water. Similarly a goldfish dipped into liquid air until brittle may revive when placed back in the water (Luyet, '38).

It has been established by many authors that a variety of frogs and fish can survive if *partly* frozen, but never if totally frozen (Marion, 1891; Cameron and Brownlee, '13a and b; Cameron, '14; Martin, '15; Weigmann, '29, '36; Borodin, '34; and others; for a complete reference list see Luyet and Gehenio, '40). An excised frog heart will survive — 2.5°, but not — 3.0°C. (Cameron, '14). Freezing of frog muscle fibers likewise always produced their death (Chambers and Hale, '32).

Borodin ('34) had blackfish sent from Alaska to Harvard University where he subjected them to freezing experiments. They survived 40 minutes' exposure to — 20°, but died from 60 minutes' exposure.[10] The fish were killed when frozen in water.

In the middle of April, 1948, a good supply of blackfish were taken at Bethel on the Kuskokwim River. The following experiments were performed on the spot. Six fishes were placed out of water, but wet, on a dish in a refrigerator at — 6°. After 12 hours ice could be seen in the eyes of the fish. Upon thawing in water, in which the fish now floated on account of the internal ice formation, the fish remained in the rigor position, dead, and with bubbles of gas frozen out in the anterior eye chambers. Another six fishes were placed in a wet towel for six hours at — 12°. All died in rigor and floated when thawing, until the internal ice was melted, when they again sank. Other fishes, used as controls, lived for several days when kept out of water in a wet towel above freezing.

Back at Point Barrow a few days later several more experiments were made. Eight fish were put under a screen in a bowl of water and frozen into a block of ice at about — 20°. When thawed after four hours' freezing they usually oozed blood, and most of them had cataracts and bubbles in their eyes. All were dead in rigor.

These findings fully confirm Borodin's experiments that the *Dallia* certainly cannot be anywhere near completely frozen and still survive. However, the blackfish, like man and other animals, can survive being partially frozen, and the degree of freezing which it can stand may differ vastly from that which is possible for many other vertebrates. A series of experiments was performed where the fish was only partially frozen, showing its remarkable tenacity of life, even though damaged beyond survival.

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[10] He also reported that a blackfish kept in a current of 6.5° water had a body temperature of + 22°. Since maintenance of such a gradient is incompatible with the fact that *Dallia* has a perfectly ordinary resting metabolic rate (Scholander, Flagg, Walters and Irving, '53), we performed temperature measurements on 36 fish at four temperature levels between 1° and 20°, and found in all cases that the body temperature ran within 0.1° of the water temperature.

Freezing of the tail and body. Fishes were placed in a thin rubber sack and dipped from the tail up to the pectoral fins in Prestone at — 26°. After a few minutes of this freezing they were thawed. Their backs had now a strong dorsal flexion, and all circulation in the frozen fins had stopped. The fish swam about by means of their pectoral fins, but they died within a few days. Two survived as long as five days before they died. Circulation in the frozen fins was never reestablished.

Freezing of the head. Five fishes were frozen in the same way from the nose to gill covers. Upon thawing there were cataracts of the lens and air bubbles in the eye chambers. The fish swam around the next day, in spite of a circulationless dead head. There was no circulation in the iris capillaries and there were no opercular movements, and all fish died in the course of three days.

Freezing of the heart. Four hearts were excised and frozen at — 6° for an hour and thawed. The beating had stopped, but faint contractions could be elicited by pricking. At — 26° the hearts were killed. A fish frozen at — 26° and thawed was dead, and the exposed heart did not respond to artificial stimulation.

Actual freezing of the tissues of the blackfish causes irreversible damage, and it is out of the question that the fish as a whole can stand being frozen.

If unfrozen but enclosed in a block of ice, the survival of the fish will depend upon how long it can remain virtually without gas exchange. This was tested. Two fishes were placed each in a tight-fitting test tube filled completely with water and stoppered. They were put in a + 1° water bath. Both fishes died after two days. Hence even if unfrozen but trapped in a tight-fitting ice chamber the fish would die from asphyxia in a few days.

We may conclude this chapter emphasizing that our observations have in no sense defamed the blackfish. Every winter they are still being thrown hard frozen to the ravenous dogs of the Yukon and Kuskokwim valleys, and indeed a fortunate observer may still, like Turner, *see* the dogs throw up the fish alive, provided only that the fish were not *too* hard frozen.

SUMMARY

A quantitative study has been made of certain aspects of the physiological processes in frozen arctic plants and animals.

1. Seven out of eight species of vascular plants were found to be dehydrated during the winter as compared with their summer state. The total winter moisture amounted to some 25–80% of the summer moisture. Live chironomids frozen into the ice were 10% to 20% dehydrated.

2. Chironomid larvae spend the winter frozen into the ice or mud of lakes. When out of water they can be thawed and refrozen in the laboratory many times without injury. The amount of ice present in them was determined in relation to temperature using a new micro flotation method. Their body fluids freeze to such an extent that the concentration of the solids to the unfrozen water remains approximately proportional to the temperature below 0°. The quantity of unfrozen water decreases, in other words, approximately in inverse proportion to the temperature. The unfrozen water is not held in a supercooled state, as the freezing curves are reversible, and freezing cannot be induced by mashing the partially frozen animal. Ice determinations in a lichen are less quantitative, but show the same trends.

3. Respiratory gas exchange was determined at graded temperatures down to -26°C. in five species of vascular plants, four species of lichens, and in chironomid larvae. The determinations were made by enclosing the material in chambers at the desired temperature until the gas exchange could be determined by gas analysis. In all material there was a precipitous drop in the respiration when freezing set in. The Q_{10} changed

abruptly from some 2–5 above freezing to some 20–50 below freezing. The curve down to — 26° ran in most cases perfectly regularly with a quite constant Q_{10}. In the chironomids, where the ice formation is quantitatively known, we may say that the respiration below freezing is the resultant of three factors: (1) the Q_{10} *per se*, (2) the dehydration, and (3) the diffusion barrier set up by the ice crystals. The quantitative importance of these factors is discussed. The extremely high metabolic Q_{10} of a frozen organism seems to carry in itself the potentiality for extremely long survival time even at moderately cold but persistent temperatures.

4. Parts of extremely cold-hardy lichens and twigs of shrubs and trees were subjected to — 183°C., the temperature of liquid oxygen. They all respired after the treatment, but the vascular plants were unable to develop or produce leaves, and hence were damaged. Whether the lichens were damaged or not could not be determined.

5. Many animals and plants spend the winter frozen into ice at temperatures where they would normally respire at a measurable rate. We have investigated the possibility of gas exchange through the ice. The solubility of air in ice, if any, was found to be less than one thousandth of that in water. CO_2 diffused through a piece of ice 0.10 mm thick at a rate of 0.006 to 0.01 mm^3 per square centimeter per millimeter thickness per atmosphere per hour, which is some 40,000 to 70,000 times slower than through water. Oxygen diffused even more slowly through ice. At low temperatures and at shallow depths in the ice, it seems that adequate gas exchange can take place for an animal like a chironomid. Normally, however, in thick ice and at higher temperatures, the situation must be considered practically completely anaerobic.

6. Freezing experiments were performed on the Alaska blackfish *(Dallia pectoralis)*, which is commonly reported to stand freezing. Our experiments confirm Borodin's ('34) conclusions that total freezing in or out of water invariably kills the fish. Partial freezing of the tail irreversibly damaged this

part and killed the fish if the freezing was extensive. Freezing of the head always resulted in cataracts and gas bubbles in the eyes, and the rest of the animal died in a few days. The heart, *in situ* or excised, does not survive being frozen. Confined in water in a tight-fitting test tube at + 1° a *Dallia* would die in three days. Thus, if enclosed in a tight-fitting ice chamber the fish could only survive a few days.

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